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Role of a Mitogen-activated Protein Kinase Pathway in the Induction of Phase II Detoxifying Enzymes by Chemicals*

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Mitogen-activated protein kinase (MAPK) cascades are activated by diverse extracellular signals and participate in the regulation of an array of cellular programs. In this study, we investigated the roles of MAPKs in the induction of phase II detoxifying enzymes by chemicals. Treatment of human hepatoma (HepG2) and murine hepatoma (Hepa1c1c7) cells with *tert*-butylhydroquinone (tBHQ) or sulforaphane (SUL), two potent phase II enzyme inducers, stimulated the activity of extracellular signal-regulated protein kinase 2 (ERK2) but not c-Jun N-terminal kinase 1. tBHQ and SUL also activated MAPK kinase. Inhibition of MAPK kinase with its inhibitor, PD98059, abolished ERK2 activation and impaired the induction of quinone reductase, a phase II detoxifying enzyme, and antioxidant response element (ARE)-linked reporter gene by tBHQ and SUL. Overexpression of a dominant-negative mutant of ERK2 also attenuated tBHQ and SUL induction of ARE reporter gene activity. Interestingly, although expression of Ras and its mutant forms showed distinct effects on basal ARE reporter gene activity, they did not affect the activation of reporter gene by the inducers. Furthermore, a dominant-negative mutant of Ras had little effect on ERK2 activation by tBHQ and SUL, implicating a Ras-independent mechanism. Indeed, both tBHQ and SUL were able to stimulate Raf-1 kinase activity *in vivo* as well as *in vitro*. Thus, our results indicate that the induction of ARE-dependent phase II detoxifying enzymes is mediated by a MAPK pathway, which may involve direct activation of Raf-1 by the inducers.

Mitogen-activated protein kinases (MAPKs),[1] which belong to the superfamily of serine/threonine kinases, are evolutionarily conserved in all eucaryotes and play a central role in transducing various extracellular signals into the nuclei (1). A typical MAPK cascade consists of three kinases: a MAPK kinase kinase, which phosphorylates and activates a MAPK kinase, which, in turn, phosphorylates and activates MAPK (2). A well established MAPK pathway is the Ras-dependent activation of extracellular signal-regulated protein kinases (ERKs) (3). In this pathway, activated Ras recruits Raf (a MAPK kinase kinase) to the membrane, resulting in activation of Raf. The activated Raf then phosphorylates and activates MEK (a MAPK kinase), which directly activates ERK through dual phosphorylation on threonyl and tyrosyl residues within the tripeptide motif TEY. Parallel to ERK pathway, c-Jun N-terminal kinase (JNK) is regulated by a distinct module consisting of MEKK1/ASK/TAK-MKK4/MKK7-JNK (4), which is farther regulated by the small GTPases, Rac1 or Cdc42 (5). ERK and JNK are often responsive to different extracellular signals (6, 7). However, they can also be activated by the same stimuli such as mitogenic signals, growth factors, oncogenic Ras (8, 9), stress signals, UV radiation, and oxidative stress (10, 11). Once activated, ERK and JNK can phosphorylate a number of cytosolic proteins and transcription factors such as c-Jun, ATF2, and ternary complex factors, resulting in the enhancement of their transcriptional activities and activation of dependent genes (12). Considering the general involvement of MAPK pathways in cellular responses to various stimuli, we examined the roles of these kinases in the induction of phase II detoxifying enzymes by chemicals.

Phase II detoxifying enzymes include NAD(P)H:quinone oxidoreductase/DT-diaphorase (QR), glutathione *S*-transferases (GSTs), UDP-glucuronosyl transferases, and epoxide hydrolases. These enzymes are capable of converting the reactive electrophiles to less toxic and more readily excretable products, thus protecting cells against various chemical stresses and carcinogenesis (13–15). Biochemical and genetic studies revealed that the induction of phase II detoxifying enzymes by various chemicals occurs at the transcriptional level and is regulated by a *cis*-acting regulatory element, defined as antioxidant responsive element (ARE) or electrophile-responsive element. This regulatory element was first detected in the 5′-flanking region of the rat and mouse GST Ya subunit gene (16–18) and human QR genes (19, 20) and is also expected to be present in the promoters of epoxide hydrolase and UDP-glucuronosyl transferase genes. Because the ARE core sequence (GTGACnnnGC) is similar to AP-1-binding site (TGACTCA), it has been suggested that AP-1 may be the activator of ARE. Indeed, the components of AP-1 complex, such as c-Jun and c-Fos, are found to bind to ARE sequence, and overexpression of c-Jun leads the induction of ARE-dependent genes (21–24). However, several independent studies indicate that the major

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[1] The abbreviations used are: MAPK, mitogen-activate protein kinase; tBHQ, *tert*-butylhydroquinone; SUL, sulforaphane; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK kinase; ARE, antioxidant responsive element; QR, quinone oxidoreductase; GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase; MBP, myelin basic protein; HA, hemagglutinin; UVC, ultraviolet c.

ARE-binding proteins that mediate the induction of detoxifying enzymes may not be the AP-1 proteins (25–28). Although the identity of ARE-binding proteins remains to be characterized, activation of the ARE-protein complex by phase II enzyme inducers is believed to be regulated by signal transducing kinase cascades (29, 30). In this study, we identified ERK2 kinase pathway to be involved in the ARE-mediated induction of phase II detoxifying enzymes by *tert*-butylhydroquinone (tBHQ) and sulforaphane (SUL). Furthermore, we showed that this induction may involve direct activation Raf-1 by the inducers, thus implicating the existence of a novel Ras-independent pathway for Raf-1 activation.

MATERIALS AND METHODS

Cell Culture, Antibodies, DNA Plasmids, and Chemicals—HepG2 and Hepa1c1c7 cell lines (obtained from American Type Culture Collection, Manassas, VA) were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 2.2 g/liter sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were normally starved overnight in serum-free medium before treatment, unless otherwise indicated. Rabbit anti-ERK2 and anti-Raf-1 polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-HA monoclonal antibody (12CA5) was purchased from Boeringer Mannheim. Rabbit anti-JNK1 antiserum (Ab101) was described previously (31). GST-c-Jun (1–79)-expressing plasmid was kindly provided by Dr. Karin (University of California, San Diego, CA). GST-c-Jun fusion protein was purified from *Escherichia coli* lysates with aid of glutathione-Sepharose beads (Amersham Pharmacia Biotech). Construct pARE-CAT containing a single copy of 41-base pair rat GST-Ya subunit ARE (5′-GAGCTTGGAAATGGCATTGCTAATGGTGACAAAGCAACTTT) and a minimal GST-Ya promoter was a gift from Dr. Rushmore (Merck Research Laboratory, West Point, PA). ARE-luciferase reporter construct, pTI-ARE-luciferase, which contains a single copy of 41-base pair mouse ARE (5′-TAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTT) and minimal TATA-Inr promoter) was obtained from Dr. Fahl (University of Wisconsin, Madison, WI). pLNCAL7 expression constructs for HA-tagged ERK2(WT) and dominant-negative mutant ERK2(KR) have been described previously (32). pZIP retrovirus constructs of *ras*(WT), *ras*(61L), and *ras*(17N) were constructed as described previously (33). The specific MEK-1 inhibitor (PD98059) and the Raf-1 substrate (inactive MEK fusion protein) were purchased from New England Biolabs Inc. (Beverly, MA). SUL was purchased from LKT Laboratories (St. Paul, MN). tBHQ was purchased from Aldrich. Myelin basic protein (MBP) was purchased from Sigma. [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from NEN Life Science Products.

Immunocomplex Kinase Assays of ERK2, JNK1, and Raf-1 Activities—After treatment with SUL or tBHQ (both agents were dissolved in Me_2SO), and cells were washed twice with ice-cold phosphate-buffered saline and harvested in lysis buffer containing 10 mM Tris-HCl, pH 7.1, 50 mM NaCl, 50 mM NaF, 30 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 2 mM iodoacetatic acid, 5 μM zinc chloride, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. Cell lysates were homogenized by passing through a 23-G needle three times and left on ice for 15 min. The homogenates were then centrifuged at high speed for 15 min at 4 °C. Endogenous ERK2, JNK1, or Raf-1 in the supernatants were immunoprecipitated with the respective antibodies and assayed for kinase activity by the method described previously (31). Briefly, following immunoprecipitation, the immunocomplex was washed twice with lysis buffer and twice with kinase assay buffer containing 20 mM HEPES, pH 7.9, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM Na_3VO_4, 50 mM β-glycerophosphate, and 10 mM ρ-nitrophenyl phosphate. Kinase reaction was initiated by resuspending the immunopreciptate in a 30-μl kinase assay buffer supplemented with 2 μCi of [γ-^{32}P]ATP, 20 μM ATP, and 2 μg of the indicated substrates. After incubation for 15 min in ERK and Raf-1 assays or for 30 min in JNK assay at 30 °C, the reaction was terminated with Laemmli's buffer. Samples were heated to 95 °C for 5 min and analyzed by electrophoresis. The phosphorylated substrates were visualized by autoradiography, and quantitated with a phosphorimager (AMBIS, Inc., San Diego, CA).

MAPK Kinase Assay—After treatment, cell lysates were prepared as described in immunocomplex kinase assays. Total activity of MAPK kinase 1 and MAPK kinase 2 (MEK1/2) in cell lysates was determined using an inactive $p42^{MAPK}$ mutant K52R as substrate as described previously (34). Briefly, equal portions (about 2 μg of protein) of cell lysates was incubated with 2.5 μg of K52R in a 30-μl kinase assay buffer with addition of 2 μCi of [γ-^{32}P]ATP and 25 μM ATP. The kinase reaction was terminated with Laemmli's buffer 15 min after incubation at 30 °C. Phosphorylated K52R was resolved on 10% SDS-polyacrylamide gels and visualized by autoradiography.

Transfection and Assays of Reporter Gene Activity—HepG2 cells were plated in six-well plates 24 h before transfection at a density of 1.5×10^5 cells/well. Cells were transfected with different plasmids as indicated in the figure legends using FuGENE™ 6 (0.7 μg of DNA/μl of reagent), according to the manufacturer's protocol (Roche Molecular Biochemicals). After overnight incubation with transfection mixture, cells were cultured in fresh medium containing 0.5% fetal bovine serum for 12 h prior to drug treatment. The β-galactosidase activity was determined as described previously (35). For CAT activity assay, transfected cells were washed twice with ice-cold phosphate-buffered saline after drug treatment and harvested in lysis buffer provided by manufacturer (Promega, Madison, WI). 10 μg of protein, as determined by Bradford assay (Bio-Rad), was incubated with reaction buffer for 60 min at 37 °C. The acetylated products of [^{14}C]chloramphenicol were separated by TLC, visualized by autoradiography, and quantitated by Biological Image Analysis (AMBIS, Inc., San Diego, CA). All CAT activities were normalized against β-galactosidase activity. Luciferase activity was determined according to the protocol provided by manufacturer (Promega). Briefly, after drug treatment, cells were washed twice with ice-cold phosphate-buffered saline and harvested in reporter lysis buffer. Following brief centrifugation (5 s) at high speed, the supernatant was transferred to a new tube, and 20 μl of cell lysate was assayed for luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity was normalized against β-galactosidase activity yielding a final value of relative light units/β-galactosidase units.

Quinone Reductase Activity Assay—After treatment, Hepa1c1c7 cells were washed twice with ice-cold phosphate-buffered saline and harvested in a buffer containing 25 mM Tris-HCl, pH 7.4, and 125 mM sucrose. Cell suspension was sonicated for 5 s and left on ice for 10 min. The homogenates were centrifuged at 13,000 $\times$ *g* for 20 min at 4 °C. Supernatants were transferred to the new tubes, and protein concentration was determined by Bradford assay (Bio-Rad). Quinone reductase activity was determined by measuring the reduction of 2,6-dichloroindophenol (36). Approximately 5 μg of total cytosolic protein was added to the cuvette containing 1 ml of assay buffer (25 mM Tris-HCl, pH 7.4, 60 μg bovine serum albumin, 5 μM FAD, 0.2 mM NADH, 80 μM 2, 6-dichloroindophenol, and 0.01% Tween 20). Reaction was performed for 5 min at room temperature and terminated with 30 μM dicumarol. The absorbance of reaction mixture at 600 nm was read on a spectrophotometer, and QR activity was expressed as nmol of 2,6-dichloroindophenol reduced per min per mg of protein.

Western Blotting—Cell lysates were prepared as described in immunocomplex kinase assays. 25 μg of protein was resolved with 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difuoride membrane using a semi-dry transfer system (Fisher). Membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 20 mM Tris-HCl, pH 7.4, 8 g/liter NaCl, and 0.2 g/liter KCl for 1 h at room temperature, followed by incubation with 1 μg/ml primary antibodies in Tris-buffered saline overnight at 4 °C. Membrane was washed three times with Tris-buffered saline and blotted with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Membrane was again washed three times with Tris-buffered saline and analyzed by the ECL system (Amersham Pharmacia Biotech).

RESULTS

SUL and tBHQ Stimulate ERK2 Activity in Human HepG2 and Mouse Hepa1c1c7 Hepatoma Cell Lines—In the previous studies, we have shown that tBHQ induced ERK2 activity in a time- and dose-dependent manner in HepG2 cells (37). To study the activation of ERK2 by SUL, we treated HepG2 cells with various concentrations of SUL. The endogenous ERK2 activity was determined by *in vitro* immunocomplex kinase assays. As shown in Fig. 1*A*, ERK2 activity began to increase at 5 μM of SUL. A maximal activity (approximately 8-fold over the control cells) was seen at 50 μM. The stimulated ERK2 activity began to decline when the concentration of SUL reached 100 μM, indicating that ERK2 activation by SUL was a dose-dependent event. The decreased ERK2 activation at relative high concentrations of SUL, such 100 μM, seemed not due to the

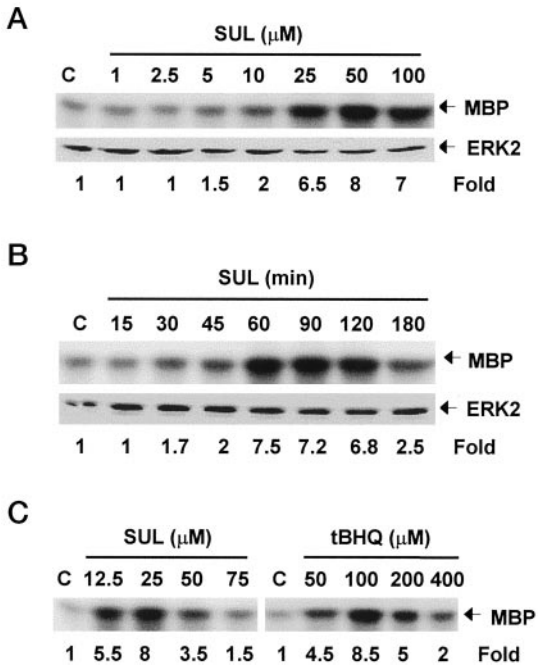

FIG. 1. **Activation of ERK2 by SUL and tBHQ.** *A*, dose response of ERK2 activation by SUL. After overnight serum starvation, HepG2 cells were treated either with different concentrations of SUL for 1 h or with 0.1% Me_2SO as control. The endogenous ERK2 activity was determined by immunocomplex kinase assays as described under "Materials and Methods" with MBP as substrate. The protein level of ERK2 was determined by Western blotting. *B*, time course of ERK2 activation by SUL. HepG2 cells were either treated with 0.1% Me_2SO as control or with 25 μM SUL for different times as indicated. ERK2 activity and protein level were determined as in *A*. *C*, dose-dependent activation of ERK2 by SUL and tBHQ in Hepa1c1c7 cells. After treatment for 1 h with different concentrations of SUL or tBHQ, cells were harvested for ERK2 activity assay as in *A*. The data presented are the means of three independent experiments.

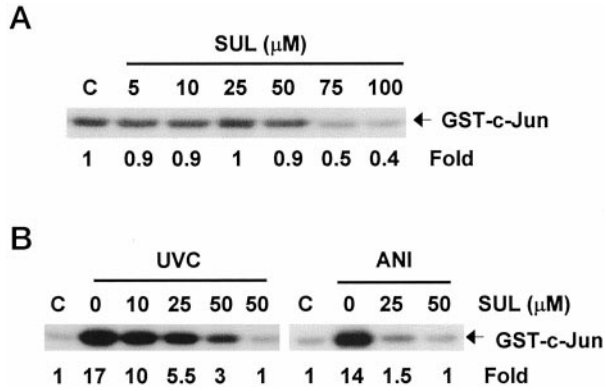

FIG. 2. **Effect of SUL on JNK1 activity.** *A*, dose-dependent effect of SUL on JNK1 activity. Serum-starved HepG2 cells were treated with various concentrations of SUL for 2 h and harvested. JNK1 activity was determined by immunocomplex kinase assays with GST-c-Jun (1–79) as substrate. *B*, dose-dependent inhibition of UVC- or anisomycin-induced JNK1 activity by SUL. Serum-starved HepG2 cells were pretreated with the indicated concentrations of SUL for 1 h before stimulation with UVC (80 J/m^2) or 10 μg/ml of anisomycin (*ANI*) for 30 min. Cells were harvested and assayed for JNK1 activity as in *A*. The experiment was repeated for three times.

toxic effect of this compound, because no morphological change or cell death was observed when ERK2 activity was measured (data not shown).

ERK2 activation by SUL in HepG2 cells was also time-dependent (Fig. 1*B*). The induced ERK2 activity appeared at 30 min, reached the maximum between 1 and 2 h after treatment with SUL, and then declined. Interestingly, the time course of ERK2 activation by SUL was very similar to that of ERK2 activation by tBHQ (37), suggesting that two inducers may regulate ERK2 activity through a common pathway. When Western blotting was performed, no change in the protein level of ERK2 was observed throughout the dose response as well as time course studies (Fig. 1, *A* and *B*), indicating that the induction of ERK2 activity resulted from the phosphorylation of pre-existing ERK2 molecules rather than *de novo* protein synthesis.

We also examined ERK2 activation in Hepa1c1c7 cells, which, like HepG2 cells, have been widely used for the study of phase II enzyme induction (38). As shown in Fig. 1*C*, both SUL and tBHQ stimulated ERK2 activity. The activation of ERK2 was dose-dependent, with a maximal activity seen at 25 μM of SUL or at 100 μM of tBHQ.

SUL Does Not Stimulate JNK Activity and Instead Inhibits UVC- and Anisomycin-induced JNK Activation—After demonstration of ERK2 activation, we examined the involvement of JNK1, another member of MAPK family. As shown in Fig. 2*A*, SUL did not stimulate JNK1 activity; instead, relatively high concentrations of SUL reduced the JNK1 activity to the levels much lower than that seen in control cells (0.1% Me_2SO-treated cells). Furthermore, SUL, at concentrations that induced ERK2 activity, inhibited JNK1 activation by UVC and anisomycin (Fig. 2*B*). These results, together with our previous observation that tBHQ weakly stimulated JNK1 activity in HepG2 cells (37), suggest that the JNK pathway may not be involved or at least are not essential in tBHQ- or SUL-induced cell signaling that leads to the induction of gene expression.

ERK2 Activation by tBHQ and SUL Is MEK-dependent—We have previously shown that ERK2 activation by tBHQ requires the involvement of an upstream signaling kinase MAPK/ERK kinase (MEK) (37). To determine whether ERK2 activation by SUL also requires MEK, we measured the MEK activity in SUL-treated HepG2 cells using an inactive kinase ERK2(K52R) as substrate. As shown in Fig. 3*A*, SUL stimulated phosphorylation of K52R in a dose-dependent manner similar to that seen in ERK2 activity assay. This result indicates that MEK was activated in the cells treated with SUL.

To provide further evidence for the involvement of MEK in ERK2 activation by SUL, we took advantage of a recently identified specific MEK inhibitor, PD98059 (39). In solvent (0.1% Me_2SO)-pretreated HepG2 cells, SUL strongly induced ERK2 activity; however, preincubation with 25 μM or 50 μM PD98059 completely blocked SUL activation of ERK2 (Fig. 3*B*). PD98059 alone had no detectable effect on ERK2 activity compared with the control cells. In Hepa1c1c7 cells, PD98059 also blocked ERK2 activation by SUL (Fig. 3*C*). Taken together, these data demonstrate that SUL, like tBHQ, induced MEK-dependent activation of ERK2.

Inhibition of ERK2 Activation Attenuates the Induction of Quinone Reductase Activity by tBHQ and SUL—tBHQ and SUL induce many phase II detoxifying enzymes, such as GST, QR, and UDP-glucuronosyl transferase-glucuronosyltransferase. Experiments with Hepa1c1c7 cells showed that QR induction is a useful indicator of overall phase II enzyme induction (15, 41, 42). To provide first evidence for the involvement of ERK2 pathway in the regulation of phase II enzyme induction by SUL and tBHQ, we examined the effect of PD98059 on QR activity induced by tBHQ and SUL in Hepa1c1c7 cells. As shown in Fig. 4, tBHQ (50 μM) and SUL (12.5 μM) significantly stimulated QR activity. Pretreatment with PD98059 caused a dose-dependent inhibition of tBHQ- and SUL-induced QR activity. However, PD98059 alone only slightly decreased the basal QR activity. These data suggest that the induction of phase II detoxifying enzymes by tBHQ

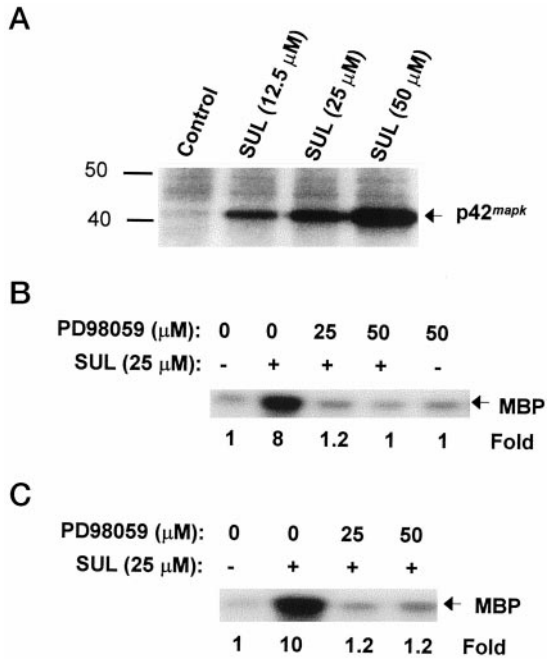

FIG. 3. **MEK-dependent activation of ERK2 by SUL.** *A*, activation of MEK by SUL. Serum-starved HepG2 cells were either treated with various concentrations of SUL or with 0.1% Me_2SO (*Control*) for 1 h. MEK activity was determined by the phosphorylation of a kinase inactive ERK2 mutant protein $p42^{MAPK}$ as described under "Materials and Methods." *B*, blockade of SUL-induced ERK2 activity by a MEK inhibitor, PD98059, in HepG2 cells. Serum-starved HepG2 cells were pretreated with the indicated concentrations of PD98059 for 1 h before exposure to 25 μM SUL for an additional 1 h. ERK2 activity was determined using MBP as a substrate. *C*, blockade of SUL-induced ERK2 activity by a MEK inhibitor, PD98059, in Hepa1c1c7 cells. Serum-starved Hepa1c1c7 cells were pretreated with PD98059 prior to stimulation with SUL as in *B*. Cells were then harvested for ERK2 activity assay. Similar results were obtained in at least three different experiments.

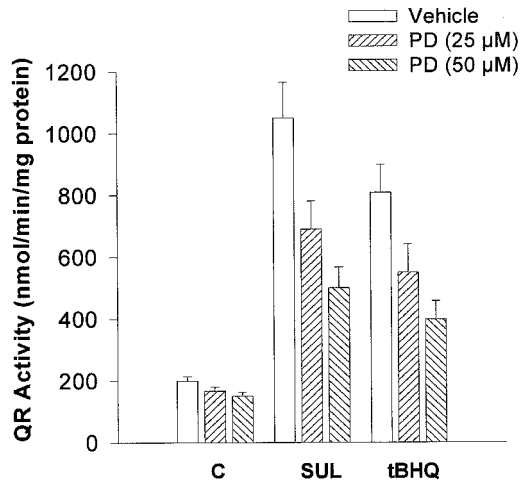

FIG. 4. **Inhibition of SUL- and tBHQ-induced QR activity by PD98059.** Hepa1c1c7 cells were either pretreated with vehicle (0.1% Me_2SO) or with the indicated concentrations of PD98059 for 1 h, prior to incubation with 12.5 μM SUL or 50 μM tBHQ for 24 h in the continuing presence of PD98059. Cells were harvested and assayed for the QR activity as described under "Materials and Methods." The data presented are averages of four independent experiments.

and SUL may be regulated by an ERK-dependent mechanism.

Inhibition of ERK2 Activation Diminishes the Induction of ARE-dependent CAT Activity by tBHQ and SUL—Previous studies have shown that the induction of phase II detoxifying enzymes by tBHQ and SUL is mediated by ARE (17, 18, 41, 43). Thus, we decided to examine whether the inhibitory effect of PD98059 on tBHQ- or SUL-induced QR activity was due to the inhibition of ARE-mediated gene expression. HepG2 cells were transiently transfected with a plasmid construct containing a single copy of 41-base pair ARE enhancer linked to the GST Ya minimal promoter (base pairs −164 to +66)-CAT gene. Exposure of transfected HepG2 cells to 25 μM SUL caused a time-dependent induction of CAT activity (Fig. 5*A*). The induced CAT activity was seen as early as 4 h, immediately following the peak of ERK2 activation (Fig. 2*B*). Pretreatment with PD98059 (25 μM) substantially reduced the induction of reporter gene activity by SUL (Fig. 5*A*). tBHQ (100 μM) also strongly induced CAT activity, which was inhibited by PD98059 in a dose-dependent manner (Fig. 5*B*). PD98059 alone only slightly decreased CAT activity as compared with the control cells (treated with 0.1% Me_2SO). When the cells transfected with a CAT construct containing GST Ya minimal promoter but lacking ARE enhancer were stimulated with SUL or tBHQ, no induction of CAT activity was observed (Fig. 5*C*). These data provide strong evidence for the role of ERK2 pathway in ARE-mediated phase II enzyme induction by SUL and tBHQ.

Interestingly, SUL-stimulated ERK2 activity was transient, peaking at 90 min (Fig. 1*B*), whereas the stimulated ARE reporter gene activity was prolonged and continued to increase even up to 24 h after stimulation with SUL (Fig. 5*A*). Although the exact reasons for such a lasting induction are not clear, it is possible that ERK2 activity may be only required for the initiation of downstream signals that are responsible for induction of ARE-dependent gene expression. Another interesting observation is that PD98059 inhibited the induction of ARE reporter gene in a time-dependent fashion. PD98059 almost completely blocked the induction of ARE activity at 4 and 8 h; however, it only partially inhibited the induction at later time points (12 and 24 h). Given that PD98059 at 25 μM exhibited similar inhibitory effect on ERK activation as higher concentration of 50 μM (Fig. 3, *B* and *C*) but showed less inhibition on ARE inhibitory (Fig. 5*B*), we speculate that a weak ERK activation (in the presence of 25 μM PD98059) may be undetectable in our system, but it sufficed to amplify the downstream effectors, contributing to such an incomplete inhibition by PD98059. Alternatively, a prolonged treatment with SUL or tBHQ may generate a second signaling event that leads to ERK-independent activation of ARE reporter gene, which could also contribute to the sustained induction as seen in this study.

Overexpression of a Dominant-negative Mutant of ERK2 Impairs ARE-mediated Induction of Luciferase Activity by SUL and tBHQ—To corroborate our experiment, we examined the effect of overexpression of a dominant-negative mutant of ERK2 on SUL- and tBHQ-induced ARE-luciferase reporter gene activity. To do so, we first tested the inducible activity of ARE-luciferase reporter gene by SUL and tBHQ. HepG2 cells were either transfected with ARE-linked luciferase reporter construct (ARE-TI-Luc) or with the construct (TI-Luc) that lacks ARE enhancer. After transfection, cells were exposed to SUL or tBHQ for 24 h, and the luciferase activity was assayed as described under "Materials and Methods." As shown in Fig. 6*A*, both SUL and tBHQ strongly induced luciferase activity in HepG2 cells transfected with ARE-TI-Luc construct but not in the cells transfected with the TI-Luc construct. Furthermore, a much lower basal luciferase activity was observed in TI-Luc-transfected cells than that in ARE-TI-Luc-transfected cells. Thus, consistent with the previous results, ARE is a regulatory sequence responsible for the high basal as well as the inducible activities of phase II enzymes. Co-transfection of ARE-luciferase reporter with a dominant-negative ERK2 mutant,

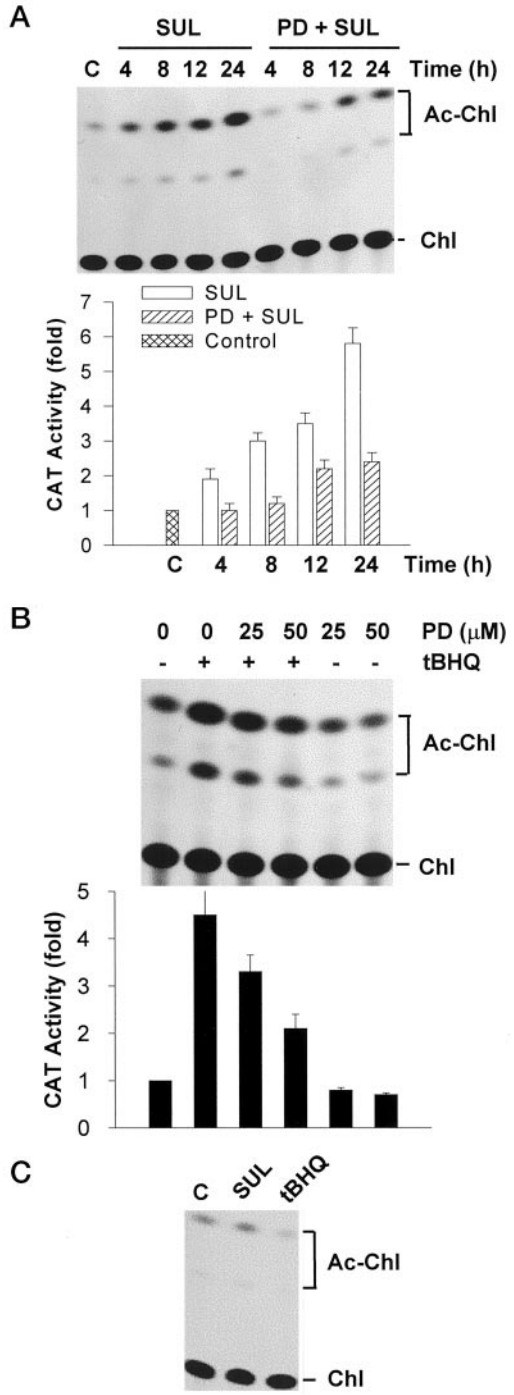

FIG. 5. **Inhibition of SUL- and tBHQ-stimulated transcription of an ARE-CAT reporter gene by PD98059.** *A*, HepG2 cells were transfected with 0.5 μg of pCH110-β-galactosidase plasmid and 1.5 μg of ARE-CAT reporter construct overnight. Transfected cells were then cultured in fresh medium containing 0.5% fetal bovine serum for 12 h and pretreated with 25 μM PD98059 or with the vehicle (0.1% Me_2SO) for 1 h, followed by exposure to 25 μM SUL for different times. CAT activity was determined as described under "Materials and Methods" and was normalized against β-galactosidase activity. The amount of CAT activity in the cells treated with vehicle alone for 24 h, as shown in *lane C*, was normalized to 1. *B*, HepG2 cells were transfected as in *A*. The transfected cells were either pretreated with the indicated concentrations of PD98059 or with the vehicle for 1 h and then exposed to 100 μM tBHQ for 24 h or left untreated. CAT activity was determined and normalized as described above. *C*, HepG2 cells were transfected with 0.5 μg of pCH110-β-galactosidase plasmid plus 1.5 μg of plasmid containing only minimal GST-Ya promoter linked to CAT gene. The transfected cells were treated with 25 μM SUL or 100 μM tBHQ for 24 h and harvested for CAT activity assay. The data presented are averages of three independent experiments done in duplicate.

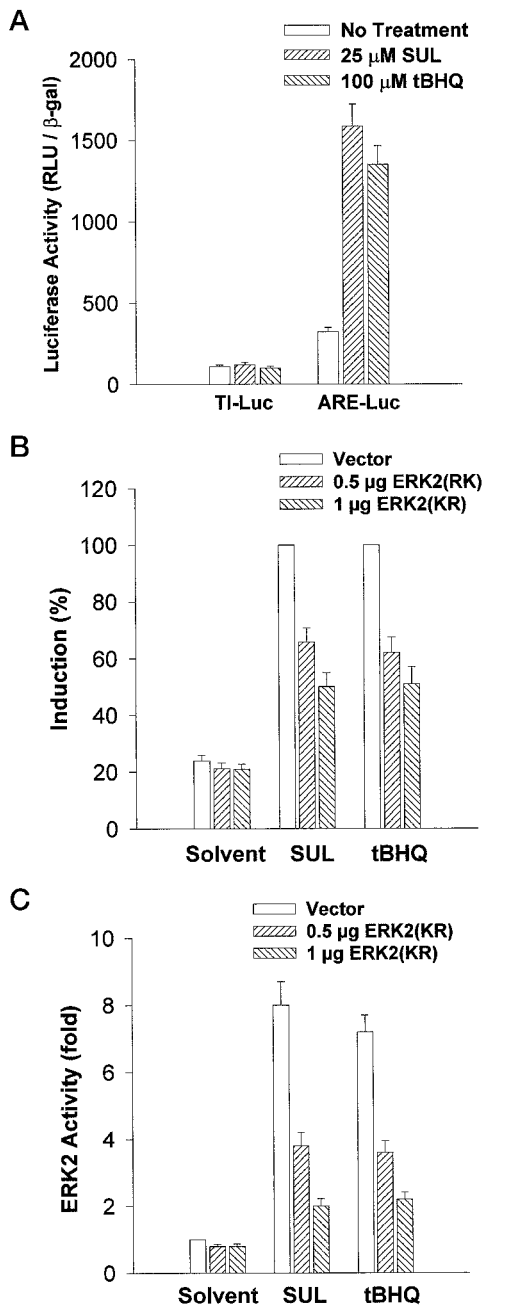

FIG. 6. **Effects of a dominant-negative mutant of ERK2 on the activation of ARE-luciferase reporter gene and ERK2 by SUL and tBHQ.** *A*, HepG2 cells were transfected with 0.5 μg of plasmid carrying ARE enhancer and synthetic TI-promoter linked to luciferase reporter gene (*ARE-Luc*) or with the reporter construct without ARE (*TI-Luc*). The plasmid (0.5 μg) encoding β-galactosidase was included as internal control of transfection efficiency. Transfected cells were treated 25 μM SUL or 100 μM tBHQ for 24 or left untreated as control. Luciferase activity was determined and normalized as described under "Materials and Methods." *B*, HepG2 cells were transfected with 0.5 μg of ARE-luciferase reporter plasmid, 0.5 μg of pCH110-β-galactosidase, and different amount of either empty expression vector or the expression vector for a dominant-negative mutant of ERK2, ERK2(KR). Transfected cells were then treated as in *A* and harvested for luciferase activity assay. The amount of luciferase activity in the cells transfected with empty expression vector and treated with SUL or tBHQ was arbitrarily set to 100%. *C*, HepG2 cells were transfected with 1 μg of HA-tagged ERK2 plasmid plus different amount of expression vector for dominant-negative mutant, ERK2(KR). Transfected cells were then treated with SUL (25 μM) or with tBHQ (100 μM) for 1 h. The exogenous ERK2 was immunoprecipitated with anti-HA monoclonal antibody (12CA5) and assayed for kinase activity using MBP as substrate. The data presented are averages of three independent experiments done in duplicate.

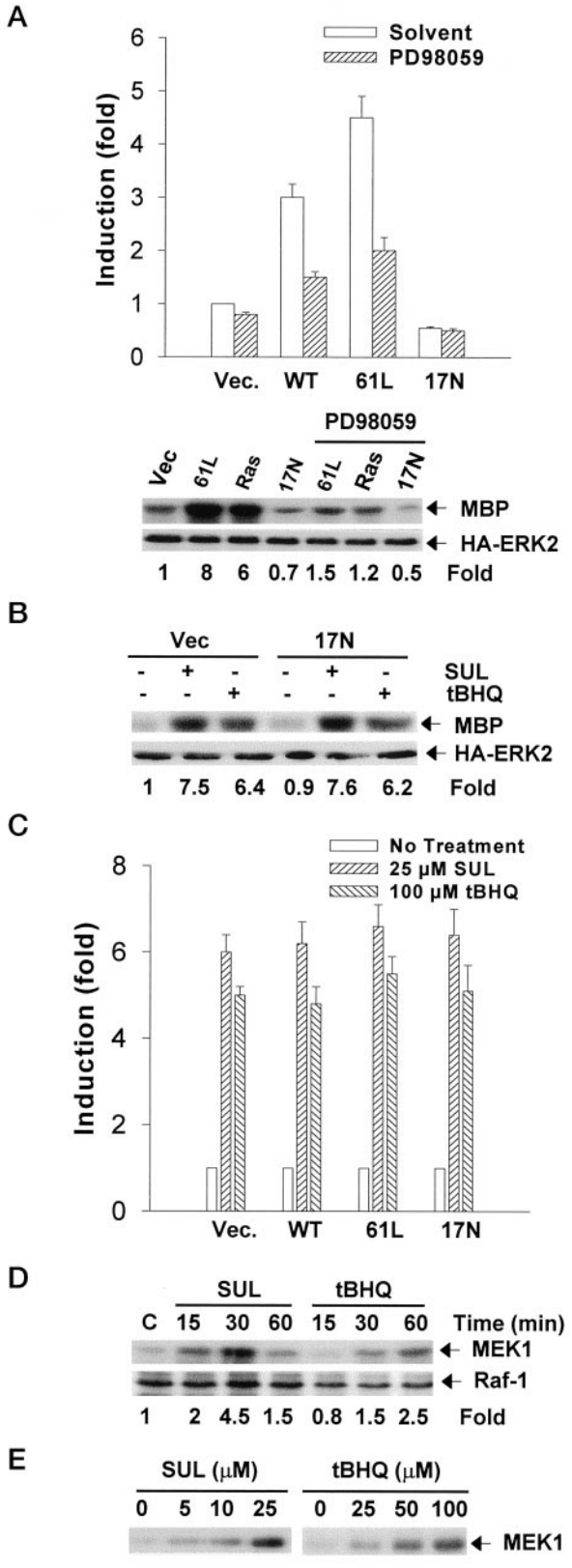

FIG. 7. **The roles of Ras and Raf-1 in the activation of ERK2 and ARE reporter gene by tBHQ and SUL.** *A*, HepG2 cells were transfected with 0.5 μg of ARE-luciferase reporter construct, 0.5 μg of β-galactosidase plasmid, and either 1 μg of empty vector or the plasmids encoding wild type Ras(WT), activated Ras(61L), or dominant-negative Ras(17N) in the presence or absence of 25 μM PD98059. Cells were harvested 24 h after transfection and assayed for luciferase activity. For ERK2 activity assay, HepG2 cells were transfected 1 μg of HA-tagged ERK2 plasmid plus 1 μg of expression vectors for Ras, Ras(61L), or Ras(17N) in the presence or absence of PD98059. Exogenous ERK2 was immunoprecipitated with anti-HA monoclonal antibody (12CA5) 24 h after transfection and assayed for kinase activity using MBP as substrate. *B*, HepG2 cells were transfected 1 μg of HA-tagged ERK2 plasmid plus 1 μg of expression vector for Ras(17N) or empty vector. 24 h after transfection, cells were treated with 25 μM SUL or 100 μM tBHQ for 1 h. Exogenous ERK2 was immunoprecipitated and assayed for kinase activity as in *A*. *C*, HepG2 cells were transfected as in *A*. After transfection, cells were either treated with 25 μM SUL or 100 μM tBHQ for 24 h or left untreated as control and then harvested for luciferase activity assay. The fold induction was calculated by using the cells transfected the corresponding vector but untreated as control. *D*, HepG2 cells were treated with SUL (25 μM) or tBHQ (100 μM) for different times. Raf-1 was immunoprecipitated with polyclonal anti-Raf-1 antibody and assayed for kinase activity using MEK fusion protein as substrate. The protein level of Raf-1 was determined by Western blotting. *E*, Raf-1 was first immunoprecipitated from untreated cells with anti-Raf-1 antibody and then incubated with different concentrations of tBHQ or SUL in a 30-μl kinase assay buffer for 30 min. Raf-1 kinase activity was measured by the phosphorylation of MEK fusion protein as in *D*. The data presented are averages or examples of three independent experiments.

ERK2(KR), significantly decreased the luciferase activity induced by SUL (25 μM) and tBHQ (100 μM) in a dose-dependent manner but had little effect on the basal activity (Fig. 6*B*). Overexpression of ERK2(KR) also significantly reduced SUL and tBHQ activation of ERK2 in a dose-dependent manner similar to that in luciferase assays (Fig. 6*C*). Therefore, these data substantiate the role or ERK2 pathway in the induction of ARE-dependent gene expression. Interestingly, ERK2(KR) also showed partial inhibition on tBHQ- and SUL-induced ARE activity (Fig. 6*B*). This could be due to the incomplete inhibition of ERK activity in ERK2(KR)-expressing cells as shown in Fig. 6*C*, although we cannot exclude the possibility that an ERK-independent pathway may exist in regulating ARE-dependent gene expression as discussed earlier.

Activation of ERK2 Pathway by tBHQ and SUL Is Independent of Ras but May Directly Involve Raf-1—Previous studies have shown that Ras is a most common upstream regulator of ERK pathway (3, 9). We therefore examined the role of Ras in tBHQ- and SUL-induced ERK2 activation and Phase II gene expression. As shown in Fig. 7*A*, overexpression of wild type Ras or an active form of Ras, Ras(61L), stimulated ARE-dependent luciferase activity and ERK2 activity, whereas a dominant-negative mutant of Ras, Ras(17N), showed the opposite effect. The activation of ARE reporter gene and ERK2 by Ras or its active form was also inhibitable by PD98059 (Fig. 7*A*). These results substantiate the role of ERK pathway in phase II gene induction. However, expression of dominant-negative Ras(17N) mutant had little effect on tBHQ- and SUL-stimulated ERK2 activity (Fig. 7*B*). Consistent with this observation, overexpression of Ras, Ras(61L), or Ras(17N) did not cause significant changes in the fold of induction of ARE reporter gene activity by tBHQ or SUL (Fig. 7*C*). These results suggest that tBHQ and SUL may activate ERK2 pathway through the component independent or downstream of Ras. Accordingly, we examined the role of Raf-1. Treatment of HepG2 cells with tBHQ or SUL stimulated Raf-1 activity, as determined by immunocomplex assays (Fig. 7*D*). More interestingly, direct incubation of tBHQ or SUL with immunoprecipitated Raf-1 also stimulated kinase activity of Raf-1 (Fig. 7*E*). This result suggests that tBHQ and SUL may directly act on Raf-1, resulting in MEK-dependent activation of ERK2 pathway.

DISCUSSION

MAPK cascades are the most conserved of signal transduction systems in all eucaryotes and have been shown to participate in cell differentiation, cell division, cell movement, and cell death (44). In this study, we found that treatment of HepG2 and Hepa1c1c7 cells with phase II detoxifying enzyme inducers

tBHQ and SUL caused activation of ERK2 kinase pathway. Inhibition of ERK2 activation by a specific MEK inhibitor, PD98059, impaired the induction of QR activity as well as the activation ARE-dependent reporter gene by tBHQ and SUL. Consistent with this, blockade of ERK2 signaling by overexpressing a dominant-negative mutant of ERK2, ERK2(KR), also attenuated the induction of ARE-dependent reporter gene activity by the inducers. Thus, this study demonstrated for the first time that ERK kinase pathway also participates in the ARE-mediated induction of phase II detoxifying enzymes.

An earlier study suggested a role of protein phosphorylation in the activation of phase II genes (30). For example, okadaic acid, a potent inhibitor of serine/threonine protein phophatases, mimics many phase II enzyme inducers to strongly stimulate ARE-dependent Ya-CAT activity, whereas genistein, a protein tyrosine kinase inhibitor, blocks the activation of ARE-dependent reporter gene by tBHQ, β-naphthoflavone, and 3-methycholathrene. The present study shows that such a role of protein phosphorylation is, perhaps, mediated by ERK2 kinase pathway. Unlike ERK2, however, no detectable JNK1 activation was observed in tBHQ- or SUL-treated cells. In fact, pretreatment with SUL inhibited JNK1 activation by UVC and anisomycin. Thus, JNK1 does not seem to play a role in the phase II enzyme induction by tBHQ- and SUL. In an independent study, we found that treatment with several phase II enzyme inducers, including tBHQ, also activated p38, another member of MAPK family. Inhibition of p38 activation by a specific p38 inhibitor, SB203580, potentiated the activation of ARE-dependent reporter gene,[2] indicative of a negative role of p38 MAPK. It is therefore conceivable that the induction of phase II detoxifying enzymes can be either positively or negatively regulated by protein phosphorylation and may involve the differential roles of the members of MAPK family.

MAPK are ubiquitously activated, but the mechanisms by which they are activated by membrane-associated upstream components vary with the cell type and stimulator, and the effectors to which they are connected likewise vary. This provides the basis of specificity, and it is important to understand the contextual diversity of the signaling. Activation of ERK pathway in response to many stimuli such growth factors and oxidative stresses has been shown to be regulated by a small GTPase, Ras (1, 3, 10, 11). In this study, we show that expression of wild type or activated form of Ras leads to the activation of ERK2 and ARE-dependent reporter gene, whereas dominant-negative mutant Ras(17N) shows the inhibitory effect. These data support the role of ERK pathway in the induction of phase II detoxifying enzymes. However, overexpression of different forms of Ras does not alter the fold of induction of ARE reporter gene activity by tBHQ and SUL. Furthermore, forced expression of dominant-negative mutant Ras(17N) has little effect on the activation of ERK2 by tBHQ and SUL, suggesting that tBHQ and SUL may activate ERK pathway through the components independent or downstream of Ras. Indeed, our results indicate that tBHQ and SUL may directly act on Raf-1, an immediate downstream effector of Ras, leading to the activation of ERK2, because incubation of tBHQ or SUL with the immunoprecipitated Raf-1 results in elevation of Raf-1 kinase activity. SUL contains an isothiocyanate group that has been shown to actively modify many proteins through the sulfhydryl groups (45). It is therefore conceivable that activation of Raf-1 may be a consequence of direct interaction of SUL and Raf-1 molecules. Unlike SUL, tBHQ may activate Raf-1 in an indirect way, probably by generation of phenoxyl free radicals, which has been previously implicated in ERK2 activation by BHA and tBHQ (37). However, the precise mechanisms by which Raf-1 is activated by tBHQ and SUL remain to be elucidated.

Activated ERK2 can phosphorylate several cytosolic and nuclear proteins, including ternary complex factor, Elk-1 (46). Phosphorylation of Elk-1 enhances its interaction with the serum response factors at the *c-fos* promoter, resulting in the induction of c-Fos, which, in turn, activates AP-1-dependent genes (12). However, the role of AP-1 in ARE-mediated activation of phase II genes remains controversial (23, 24, 27, 28, 30, 47). Besides, we did not observe any significant effect of a dominant-negative mutant of c-Jun, Tam-67, on the activation of ARE reporter gene by tBHQ and SUL (data not shown). Thus, search for the new targets of ERK2 kinase is warranted. Recently, several novel ARE-binding proteins have been identified, including the members of basic leucine zipper transcription (bZIP) factor family, Nrf1(47), Nrf2 (47, 48), and Maf (48). A novel nuclear protein, designated as ARE-BP-1, has also been described to constitutively bind to the ARE-inducible sequence, the GC box, and to be activated by tBHQ through a post-translational mechanism (49). It will be interesting to examine whether the transcription activities of these ARE-binding proteins can be regulated by ERK pathway. Most recently, a cytosolic protein, named Keap1, has been identified to suppress Nrf2 transcriptional activity by retaining Nrf2 in the cytoplasm (40). Thus, it is tempting to speculate that activation of ERK pathway may lead to the phosphorylation of Keap1 and the release of Nrf2 from Keap1-Nrf2 complex, resulting in nuclear translocation of Nrf2 and activation of ARE-dependent genes.

In summary, we have identified a signal transduction pathway that mediates the induction of ARE-dependent phase II gene expression by tBHQ and SUL. This finding advances our understanding of the regulatory mechanisms of chemical-induced phase II gene expression. Given that phase II detoxifying enzymes are induced by a variety of compounds, multiple signaling pathways may exist. A future challenge is to elucidate how ERK kinase integrates with other signaling pathways to mediate the action of various phase II enzyme inducers.

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